Growth behavior and temporal enterotoxin D expression of Staphylococcus aureus strains under glucose and lactic acid stress

Sihto, Henna-Maria ; Tasara, Taurai ; Stephan, Roger ; Johler, Sophia

Abstract: Ingestion of the staphylococcal enterotoxin D (SED) leads to staphylococcal food poisoning, the most prevalent foodborne intoxication worldwide. Patients suffer from acute signs of gastroenteritis such as violent vomiting, diarrhea, cramps, and fever. As the symptoms result in pronounced electrolyte imbalances and dehydration, the intoxication is particularly dangerous to children and the elderly. SED is formed during growth of Staphylococcus aureus in food. While growth of S. aureus is repressed by competing bacteria in most food matrices, the organism exhibits a crucial competitive growth advantage in foods with low pH or a low aw value (e.g. through high sugar concentrations). To date, the effect of these stress conditions on sed expression is unclear. The objective of this study was to determine sed mRNA expression levels of S. aureus exposed to glucose and lactic acid stress conditions similar to food production and preservation. To this end, temporal sed mRNA expression levels of three S. aureus strains grown at control conditions, glucose stress conditions (30% glucose), and lactic acid stress conditions (pH 6.0) were determined using quantitative Real-Time PCR. Under both glucose and acid stress conditions, the mean lag phase duration was prolonged and maximum cell density in late stationary phase was decreased. In addition, glucose stress slightly increased the growth rate of the tested strains and led to decreased sed expression in late stationary phase. Lactic acid stress had no statistically significant effect on sed expression. Our study provides data on the effect of critical food-related stressors on growth and SE expression of S. aureus, which can be used for risk assessment.

DOI: https://doi.org/10.1016/j.foodcont.2015.10.008

Short communication

In vitro *sed* expression under glucose and lactic acid stress

Growth behavior and temporal enterotoxin D expression of *Staphylococcus aureus* strains under glucose and lactic acid stress

Henna-Maria Sihto[a], Taurai Tasara[a], Roger Stephan[a], Sophia Johler[a,*]

[a]Institute for Food Safety and Hygiene, University of Zurich, Zurich, Switzerland, ils@fsafety.uzh.ch

***Author for correspondence:**

Institute for Food Safety and Hygiene, Vetsuisse Faculty University of Zurich, Winterthurerstr. 272, CH-8057 Zurich, Switzerland, Tel: +41-446358680, Fax: +41-446358908

ABSTRACT

Ingestion of the staphylococcal enterotoxin D (SED) leads to staphylococcal food poisoning, the most prevalent foodborne intoxication worldwide. Patients suffer from acute signs of gastroenteritis such as violent vomiting, diarrhea, cramps, and fever. As the symptoms result in pronounced electrolyte imbalances and dehydration, the intoxication is particularly dangerous to children and the elderly. SED is formed during growth of *Staphylococcus aureus* in food. While growth of *S. aureus* is repressed by competing bacteria in most food matrices, the organism exhibits a crucial competitive growth advantage in foods with low pH or a low a_w value (*e.g.* through high sugar concentrations). To date, the effect of these stress conditions on *sed* expression is unclear. The objective of this study was to determine *sed* mRNA expression levels of *S. aureus* exposed to glucose and lactic acid stress conditions similar to food production and preservation. To this end, temporal *sed* mRNA expression levels of three *S. aureus* strains grown at control conditions, glucose stress conditions (30% glucose), and lactic acid stress conditions (pH 6.0) were determined using quantitative Real-Time PCR. Under both glucose and acid stress conditions, the mean lag phase duration was prolonged and maximum cell density in late stationary phase was decreased. In addition, glucose stress slightly increased the growth rate of the tested strains and led to decreased *sed* expression in late stationary phase. Lactic acid stress had no statistically significant effect on *sed* expression. Our study provides data on the effect of critical food-related stressors on growth and SE expression of *S. aureus,* which can be used for risk assessment.

Keywords: *Staphylococcus aureus, sed*, glucose stress, lactic acid stress, stress response

1. Introduction

The Centers for Disease Control and Prevention estimate that a total of 240 000 cases of staphylococcal food poisoning (SFP) occur each year in the US, leading to hospitalization in 1000 cases and to six deaths (Scallan et al., 2011). SFP is caused by oral intake of enterotoxins such as staphylococcal enterotoxin D (SED), which are preformed by *Staphylococcus aureus* during growth. While growth of *S. aureus* is repressed by competing bacteria in food matrices, the organism exhibits a crucial growth advantage under stress conditions encountered in many foods, including mildly acidic conditions and low a_w values due to high sugar concentrations (Troller, 1986). As food processing techniques are constantly adapted to reduce costs and meet new consumer demands, knowledge on the effect of critical food-related stressors on growth and SE expression is needed to prevent outbreaks and advance quantitative microbial risk assessment (Schelin et al., 2011).

While some data on the effect of stressors on enterotoxin formation is available, most studies relied on immunological methods for enterotoxin detection (Genigeorgis, Foda, Mantis, & Sadler, 1971; Genigeorgis & Sadler, 1966). However, it was later suggested that quantification of enterotoxin expression on mRNA level represents a more useful tool to determine SFP risk (Lee, Moon, Park, Chang, & Kim, 2007), as loss of serological recognition of SEs does not equal loss of emetic activity (Bennett, 2005).

To date, the effects of glucose and lactic acid stress on *S. aureus* growth and *sed* expression are unclear. Therefore, we aimed to determine *sed* mRNA expression levels of *S. aureus* exposed to glucose and lactic acid stress conditions similar to food production and preservation.

2. Materials and methods

76 *2.1. Bacterial strains and growth conditions*

77 *S. aureus* strains used in this study are listed in Table 1. Strains were grown in LB broth
78 (Difco laboratories, Detroit, MI), in LB that was supplemented with 30% of glucose (1.6 M,
79 a_w = 0.96) (Sigma-Aldrich, Buchs, Switzerland), or lactic acid (pH 6.0, 8 mM) (Sigma-
80 Aldrich, Buchs, Switzerland). The pH in LB control (pH 7.0) and LB lactic acid media was
81 stabilized by inclusion of 100 nM 2-morpholinoethanesulfonic acid hydrate (MES hydrate)
82 (Sigma-Aldrich, Buchs, Switzerland) and the final pH was adjusted using 10 M NaOH
83 (Sigma-Aldrich, Buchs, Switzerland). Growth was monitored by viable cell count of serial
84 dilutions in 0.85% NaCl (Sigma-Aldrich, Buchs, Switzerland) on plate count agar (Oxoid,
85 Cambridge, UK). Growth parameters were calculated using DMFit 3.0 (Baranyi & Roberts,
86 1994).

87 Single colonies were transferred from 5% sheep blood agar to 5 mL of LB broth and grown
88 for 18 h (37°C, 225 rpm). Aliquots of 1 mL of the overnight cultures were centrifuged (6000
89 × *g* for 10 min) and washed twice with 0.8% NaCl to remove residual media components.
90 Day culture was inoculated with 10^{-3} dilution of washed overnight culture to result in
91 approximate cell density of 10^4 CFU/mL and incubated at 37°C, 225 rpm. Cells were
92 harvested by centrifugation (8000 × g for 5 min) at early exponential (T1), mid-exponential
93 (T2), early stationary (T3), and late stationary growth phase (T4). Cells were resuspended in
94 500 µL RNA protect bacteria reagent (Qiagen, Hombrechtikon, Switzerland), incubated at
95 room temperature for 5 min and harvested again by centrifugation (3000 × g for 5 min). Cell
96 pellets were stored at -80°C before RNA extraction. The procedure was repeated to gain two
97 independent samples of each strain, condition, and time point.

98

99 *2.2. Analysis of sed gene expression*

100 RNA was isolated and converted to cDNA by reverse transcription as previously described
101 (Sihto, Tasara, Stephan, & Johler, 2014). RNA samples were quantified and quality controlled

using the Nanodrop and Bioanalyzer instruments, respectively. The RNA integrity numbers determined for the samples using the Bioanalyzer (Agilent Technologies, Waldbronn, Germany) ranged from 7.1 to 9.1. For each sample, 100 ng of RNA was converted to cDNA using the Quantitect Reverse Transcription Kit (Qiagen). Reverse transcription was performed twice for each sample. Residual DNA contamination was ruled out in each RNA sample by including a control in which the RT enzyme (RT minus) was omitted. Quantitative PCR was performed using the SYBR Green I kit (Roche Molecular diagnostics, Penzburg, Germany) and the LightCycler 480 (Roche) instrument. Quantification was performed using the Light Cycler 480 Relative Quantification Software (Roche Molecular Diagnostics). Primer sequences, primer concentrations, and annealing temperatures are specified in Table 2. BestKeeper (Pfaffl, 2004) and geNorm (Vandesompele et al., 2002) programs were used to compare expression stability of the candidate reference genes as previously described (Sihto et al., 2014). The influence of glucose and lactic acid stress on *sed* expression in each strain was expressed as both relative expression values and fold changes in *sed* expression in early exponential, mid-exponential, early stationary, and late stationary phase relative to *sed* expression level during early exponential growth (T1) in LB in the respective strain.

2.3. Statistical analysis

Statistical analysis was performed using SPSS Statistics 22 (SPSS Inc., Chicago, IL). Results were considered significant at $p < 0.05$. Growth parameters such as lag phase duration, growth rate, and maximum cell density were compared using one-way ANOVA. Log transformed relative expression ratios of *sed* were also compared using one-way ANOVA. Strain-specific differences in *sed* expression were determined using Student's t-test.

3. Results

3.1. Reference gene validation

The expression stability of nine different reference genes was investigated under glucose and lactic acid stress in eight *S. aureus* strains. Based on BestKeeper and geNorm analysis, *gyrB*, *ftsZ*, *pyk* were the most stably expressed reference genes under glucose stress, while *rho*, *rplD, rpoB* were most stable under lactic acid stress. Three reference genes were chosen for normalization of quantification data under glucose stress (*gyrB*, *ftsZ*, *pyk*) and two reference genes under lactic acid stress (*rho*, *rplD*).

3.2. Effect of glucose stress on growth and sed expression

Growth behavior of three *S. aureus* strains in LB and LB with 30% glucose is presented in Fig. 1A. Under glucose stress, the mean lag phase duration was prolonged compared with no-stress (Δ2.55 ± 0.47 h; p = 0.00), and maximum cell density was decreased in late stationary phase (Δ0.53 ± 0.22 lg CFU mL^{-1}; p = 0.00). However, the growth rate was slightly increased under glucose stress (Δ0.09 ± 0.01 lg CFU mL^{-1} h^{-1}; p = 0.03). Glucose stress had no statistically significant effect on *sed* expression during early (T1) and mid-exponential (T2) as well as early stationary (T3) growth phases. However, we observed a trend towards reduced *sed* expression during late stationary phase, with a statistically significant reduction of *sed* expression in one strain (RKI2, p = 0.00) (Table 3, Fig. 2).

3.3. Effect of lactic acid stress on growth and sed expression

Growth behavior of three *S. aureus* strains in LB with MES and LB with lactic acid (pH 6.0) is presented in Fig. 1B. Under lactic acid stress, the mean lag phase duration was prolonged (Δ0.76 ± 0.01 h; p = 0.00) and maximum cell density was decreased in late stationary phase (Δ0.34 ± 0.09 lg CFU mL^{-1}; p = 0.00). However, the growth rate remained unchanged under both growth conditions (Δ0.00 ± 0.05 lg CFU mL^{-1} h^{-1}; p = 0.95). All strains increased *sed* expression over time. Growth under lactic acid stress did not significantly alter *sed* expression (Table 4, Fig. 3).

154

155 *3.4. Strain-specific differences in sed expression*

156 Comparing *sed* expression between strains, the food poisoning strains RKI1 and RKI2
157 tended to exhibit higher relative *sed* expression ratios than strain SAI48 associated with a case
158 of infection. When grown in LB only, *sed* expression was significantly higher during late
159 stationary growth phase (T4) in RKI2 compared to SAI48 ($p < 0.05$). Under glucose stress,
160 *sed* expression was significantly higher during early exponential phase (T1) in RKI1 ($p =$
161 0.01) and RKI2 ($p = 0.00$) compared with SAI48. Under lactic acid stress, *sed* expression was
162 significantly higher during late stationary phase (T4) in RKI2 compared to SAI48 ($p = 0.048$).

163

164 **4. Discussion**

165

166 As staphylococcal enterotoxins are extremely stable and cannot be inactivated by measures
167 such as heating of food, it is crucial to prevent enterotoxin formation by preventing *S. aureus*
168 growth in the food matrix. Various stress conditions were suggested to inhibit staphylococcal
169 growth, with glucose being reported to lead to an even more pronounced inhibition of *S.*
170 *aureus* growth than high concentrations of salts (Vilhelmsson & Miller, 2002). Vilhelmsson
171 and Miller linked catabolite repression and osmotic stress responses in *S. aureus*, predicting
172 that the use of glucose and other metabolizable carbohydrates as humectants should result in a
173 decreased growth rate in *S. aureus* (Vilhelmsson & Miller, 2002). In this study, glucose stress
174 (30% glucose) and lactic acid stress (pH 6.0) resulted in a prolonged mean lag phase and a
175 decreased maximum cell density in late stationary phase. However, the growth rate of the
176 tested *S. aureus* strains was slightly increased under glucose stress. This is consistent with
177 previous findings reporting an increased growth rate of *S. aureus* in the presence of glucose
178 (Jarvis, Lawrence, & Pritchard, 1975).

179 Previous studies suggest that the effect of a low pH on *S. aureus* growth and enterotoxin
180 formation varies with regard to the acid and strain tested (Domenech et al., 1992;
181 Genigeorgis, Foda, Mantis, & Sadler, 1971; Genigeorgis & Sadler, 1966; Rode et al., 2010;
182 Rosengren, Lindblad, & Lindqvist, 2013; Wallin-Carlquist et al., 2010). In general, growth
183 and enterotoxin formation were reported to subside at pH values below 4.0 in aerobically
184 cultured cells and pH 5.0 in anaerobically cultured cells (Domenech et al., 1992; Smith,
185 Buchanan, & Palumbo, 1983). *S. aureus* was shown to grow within the pH and lactic acid
186 ranges characteristic for the milk fermentation and cheese making process at conditions
187 simulating the initial stages of cheese production (Rosengren et al., 2013). In this study, we
188 used lactic acid and pH 6.0 to mimic conditions encountered in many food matrices
189 associated with staphylococcal food poisoning.
190 Weinrick et al. reported that a decline in pH leads to changes in gene expression including
191 reduced enterotoxin expression formerly thought to represent a glucose effect (Weinrick et al.,
192 2004; Jarvis et al., 1975). Since *S. aureus* represents one of the most osmotolerant foodborne
193 pathogens, a glucose concentration of 30% was chosen to introduce osmotic stress and to
194 mimic conditions in food products with high sugar content (*e.g.* cake or condensed milk). In
195 our study, cells grown at 30% glucose exhibited significantly decreased *sed* expression in late
196 stationary phase in strain RKI2, a trend towards decreased *sed* expression in RKI1, and
197 unaltered *sed* expression in SAI48.
198 In our study, lactic acid stress had no statistically significant effect on *sed* expression. For
199 enterotoxin SEA, it has been reported that mild lactic acid stress can increase the formation of
200 SEA (Rosengren et al., 2013). This is of particular interest, as lactic acid was reported to
201 effectively inhibit enterotoxin production (Domenech et al., 1992).
202 In our study, the two clinical strains associated with staphylococcal food poisoning were
203 shown to express *sed* at higher levels compared to the *S. aureus* strain isolated from a case of
204 infection. To date, there are no comprehensive studies demonstrating a link between *sed*

expression level and the strain origin, in particular for strains isolated from food poisoning outbreaks. However, for SEB producing strains, host factors have been shown to have an impact on toxin levels produced (Compagnone-Post, Malyankar, & Khan, 1991). Moreover, higher enterotoxin C production levels have been linked to strains of human and food origin, in contrast to animal strains with lower SEC production (Marr et al., 1993).

In conclusion, our results indicate that both glucose and pH stress have an effect on *S. aureus* growth, with a prolongation of the lag phase and decreased maximum cell densities reached in late stationary phase. In addition, glucose stress slightly increased the growth rate of the *S. aureus* strains and led to decreased *sed* expression in late stationary phase. Lactic acid stress had no statistically significant effect on *sed* expression of the tested *S. aureus* strains. The data generated in this study specifies the effect of critical food-related stressors on growth and SE expression of *S. aureus,* which can be used for risk assessment.

5. Acknowledgements

This work was supported by grants from the Swiss National Research Program 69 (40690_145211/1) and the Herrmann Herzer Foundation, and a Forschungskredit of the University of Zurich (grant no. FK-13-059).

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